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# Contribution of $O^6$-Alkylguanine and N-Alkylpurines to the Formation of Sister Chromatid Exchanges, Chromosomal Aberrations, and Gene Mutations: New Insights Gained From Studies of Genetically Engineered Mammalian Cell Lines

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$O^6$-methyl- and $O^6$-ethylguanine are the major premutagenic and precarcinogenic lesions induced in DNA by monofunctional alkylating agents, albeit formed in minor amounts. The involvement of these lesions in SCE and aberration formation is less clear. We have analyzed the contribution of $O^6$-alkylguanine to SCE and aberration formation, as well as its toxic and point mutation inducing effect in transgenic Chinese hamster ovary (CHO) cell lines that express variable amounts of human $O^6$-methylguanine-DNA methyltransferase (MGMT). Cells that overexpress MGMT (or the bacterial Ada protein) gained resistance to the formation of alkylation-induced SCEs and aberrations, as compared to MGMT deficient cells. A correlation was apparent between the level of protection for SCEs and cell killing, indicating that both phenomena are interrelated. The protective effects were dependent on the level of MGMT expression, the agent used for alkylation, and cell cycle progression. Our data suggest that at least 2 kinds of lesions are responsible for SCE and aberration formation, namely, $O^6$-alkylguanine and one or various N-alkylation products. The probability that $O^6$-methylguanine is converted into cytogenetic effects has been estimated to be about 1:30 for SCEs, and 1:147,000 and 1:22,000 for chromosomal aberrations in the first and second post-treatment mitosis, respectively. The induction of SCEs and likely also of aberrations by $O^6$-methylguanine requires two replication cycles and is supposed to involve the formation of secondary DNA lesions. Increased repair of 3-methyladenine and 7-methylguanine in CHO cells that overexpress the N-methylpurine-DNA glycosylase (MPG) after transfection with the human MPG-cDNA did not give rise to protection against methylation-induced SCEs and aberrations, probably because of incomplete excision repair. MPG overexpressing cells reacted even more sensitively to methylating agents, suggesting apurinic sites formed as a result of MPG action to be SCE and aberration-inducing lesions. 

**Key words: DNA repair, $O^6$-methylguanine-DNA methyltransferase, 3-methyladenine-DNA glycosylase, alkylating carcinogens and mutagens**

## INTRODUCTION

An understanding of the chain of events involved in the transformation of critical primary lesions into various genetic end points, including SCEs and chromosomal aberrations, requires that the primary lesions are identified. For alkylating agents which are widely distributed environmental mutagens/carcinogens and are also used for tumor chemotherapy, at least 12 sites of their reaction with DNA are known. The relative amount of DNA alkylation products induced depends on the chemical structure of the compound and the electrophilicity of the reactive alkylating species. The major alkylation site is the N7 of guanine followed by N3 of adenine, N1 of adenine, N3 of guanine, and $O^6$ of guanine. Alkylations at the DNA phosphate are also quite frequent. O-alkylated bases other than $O^6$-alkylguanine (e.g., $O^4$-alkylthymine) are generally induced at minor amounts [for review see Beranek, 1990].

With the exception of the alkylphosphotriesters, the main DNA alkylation lesions induced in DNA in mammalian cells are repaired. $O^6$-alkylguanine is repaired by the $O^6$-methylguanine-DNA methyltransferase (for mammalian cells MGMT, for E. coli the Ada and Ogt proteins) which transfers the alkyl group from the $O^6$-alkylguanine to a cystein residue in its active center. The repair protein is thereby



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inactivated, and guanine in the DNA restored [for review see Pegg, 1990]. Cell lines that do not express detectable amounts of MGMT are defined phenotypically as $Mex^-$ [Sklar and Strauss, 1981]. In these cells $O^6$-methylguanine ($O^6$-MeG) persists in DNA, indicating that damage reversal by MGMT is the main and very likely the only mechanism by which this lesion is repaired in eukaryotes.

The N-alkylpurines 7-methylguanine (7-MeG), 3-methyladenine (3-MeA), and 3-methylguanine (3-MeG) are removed from DNA by excision repair, the first step of which is mediated by the action of a N-methylpurine-DNA glycosylase (MPG). The MPG-catalyzed base hydrolysis gives rise to an apurinic (AP) site in DNA which is consecutively repaired by a concerted action of apurinic endonuclease, DNA polymerase and ligase. The mammalian MPG is comparable with regard to its broad substrate specificity to the bacterial Alk A protein [Lindahl et al., 1988]. Although MPG removes various N-methylpurines from DNA, it shows substrate preferences. Thus it removes 3-MeA at a higher rate from DNA than 7-MeG [Male et al., 1987; Ibeanu et al., 1992].

Which of the lesions induced in DNA by monofunctional alkylating agents (MAA) ultimately give rise to chromosomal aberrations and recombination events that are visibly expressed as sister chromatid exchanges (SCEs) is a matter of debate for years. Based on correlation analyses of the electrophilicity of a given agent (i.e., its selectivity to alkylate DNA at nucleophilic sites, as expressed by the Swain-scott constant s or by the initial ratio of 7-alkylguanine/$O^6$-alkylguanine in DNA) and its clastogenic potency, the conclusion has been drawn that N-alkylpurines represent the main critical lesions for induction of aberrations [Vogel and Natarajan, 1979; Vogel et al., 1990]. On the other hand, no clear-cut correlation was found between the amount of a particular DNA alkylation lesion induced, its persistence in DNA and the alkylation-induced aberration frequency [Connel and Medcalf, 1982]. The same is true for SCEs [Connel and Medcalf, 1982; Heflich et al., 1982; Morris et al., 1983].

New insights into this issue were obtained by utilizing genetically engineered cell lines that are characterized by expression of defined DNA repair functions. Although repair deficient $Mex^-$ tumor cell strains are available and have been compared to human $Mex^+$ cells with respect to their genotoxic response upon alkylation [Day et al., 1980], the conclusions are limited due to the fact that possibly other genetic changes could have accumulated in the transformed cells. Cell strains generated by DNA-mediated gene transfer have the advantage that they are isogenic, i.e. differ only in a particular function. Here we summarize our results obtained with cell strains that overexpress either MGMT or MPG upon stable transfection with the corresponding cDNA expression plasmids.

*Abbreviations*

| | |
|---|---|
| AP | apurinic sites |
| CHO | Chinese hamster ovary |
| EMS | ethyl methanesulfonate |
| ENU | N-ethyl-N-nitrosourea |
| HPRT | hypoxanthine-guanine phosphoribosyltransferase |
| MAA | monofunctional alkylating agents |
| $Mex^-$ | alkyltransferase deficient cells |
| $Mex^+$ | alkyltransferase proficient cells |
| MGMT | $O^6$-methylguanine-DNA methyltransferase |
| MMS | methyl methanesulfonate |
| MNNG | N-methyl-N′-nitro-N-nitrosoguanidine |
| MNU | N-methyl-N-nitrosourea |
| MPG | N-methylpurine-DNA glycosylase |
| $O^6$-EtG | $O^6$-ethylguanine |
| $O^6$-MeG | $O^6$-methylguanine |
| SCEs | sister chromatid exchanges |
| 3-MeA | methyladenine |
| 3-MeG | 3-methylguanine |
| 7-MeG | 7-methylguanine |

## GENETICALLY ENGINEERED CHINESE HAMSTER CELLS EXPRESSING HUMAN ALKYLTRANSFERASE

To generate strains that differ only in their capacity to repair $O^6$-alkylguanine we transfected Chinese hamster cells (strain CHO-9) that do not express MGMT activity ($Mex^-$ phenotype) with the human MGMT cDNA. The cDNA [Tano et al., 1990] was recloned into the pSV2neo expression vector and was thus brought under the control of the SV40 early promoter (the resulting plasmid was termed pSV2MGMT) [Kaina et al., 1991b]. The expression plasmid was cotransfected with pSV2neo in order to select for transfectants with the drug G418. For a control, transfections were performed with pSV2neo and salmon sperm DNA (instead of pSV2MGMT). Various stable MGMT transfectant strains have been isolated that express MGMT at variable amounts ranging from 8000 to 200000 molecules per cell. Transfection with the neo gene alone did not enhance MGMT activity above the detection level. Previously we have shown that CHO-9 cells harbour the endogenous MGMT gene [Fritz et al., 1991], the expression of which seems to be down-regulated [Tano et al., 1991; Mitra and Kaina, 1993]. The MGMT activity in the transfectants is due to expression of the human MGMT cDNA, which was shown by Northern and Western blot analyses [Kaina et al., 1991b].

## ALKYLTRANSFERASE PROTECTS AGAINST ALKYLATION-INDUCED GENE MUTATIONS

The expression of MGMT clearly reduces the gene mutation frequency of transfectants treated with MNNG or ENU (Table I). For MNNG, induced mutations were reduced by up to 98.8%, indicating that virtually all mutations were

**TABLE I. MNNG and ENU-Induced Gene Mutation Frequencies at the HPRT Locus in CHO Control (Strain Neo-C5), MGMT Transfected (Strains AT4 and AT8) and Ada Transfected (Strain ada-C4) Cells, and Estimated Level of Protection (Expressed as Percentage of Reduction of Mutation Frequency in the Alkyltransferase Transfectants)***

| Cell strain[a] | MGMT level[b] | Control | MNNG (1 μM) | MNNG-induced mutations | ENU (2 mM) | ENU-induced mutations | Level of protection (%) for MNNG | Level of protection (%) for ENU |
|---|---|---|---|---|---|---|---|---|
| CHO-9-neo-C5 | Not detectable (<2,000) | 5.8 | 31.3 | 25.5 | 171.3 | 165.5 | — | — |
| Tk40-AT4 | 58,650 | 1.2 | 1.8 | 0.69 | 34.2 | 34.2 | 97.6 | 79.3 |
| Tk40-AT8 | 41,820 | 0.3 | 0.6 | 0.3 | 37.4 | 37.4 | 98.8 | 77.4 |
| CHO-9-ada-C4 | 179,500 | 0.3 | 0.6 | 0.3 | 17.8 | 17.8 | 98.8 | 89.2 |

*Mutation frequencies are given as 6-thioguanine resistant cells per $10^5$ survivors. The mutation assay was performed as described [Kaina et al., 1991b]. Treatment with MNNG (1 μM) was virtually non-toxic for the MGMT and ada transfectants, and reduced colony formation by 70% (30% survival) in the control CHO-9-neo-C5. In case of ENU the alkyltransferase transfectants were not protected against the toxicity of the agent. A dose of 2 mM reduced colony formation in all strains by ~55%.

[a]Strain neo-C5 was transfected with the neo-gene (pSV2neo) only; strains Tk40-AT4 and Tk40-AT8 were transfected with neo and the human MGMT cDNA (plasmids pSV2neo + pSV2MGMT); strain ada-C4 was transfected with neo and the bacterial ada-gene (pSV2neo + pSV2ada); for a detailed description of these strains see Kaina et al. [1991b].

[b]Molecules per cell. For strain CHO-9-ada-C4, as well as for the other strains, the alkyltransferase activity was determined by measuring the methyl group transfer to protein from a [$^3$H]MNU alkylated DNA substrate. Since Ada has two active sites the alkyltransferase activity (as expressed by molecules per cell) was corrected by supposing half of the transferred activity to be due to removal of methyl groups from the DNA-phosphate.

brought about by lesions that are repaired by MGMT in vivo, i.e., $O^6$-MeG. $O^4$-Alkylthymine which is a mispairing lesion too, appears to be involved not significantly in MNNG but in ENU-induced mutagenesis, since Ada that repairs this lesion [Lindahl et al., 1988] is more efficient in protection than MGMT, when expressed in CHO cells (see Table I).

In contrast to MNNG, for ENU only a 77 to 89% reduction of mutation frequency was observed. The lower protective effect exerted by MGMT in case of ENU is very likely not due to the higher efficiency of ENU to induce mutations and saturation of the MGMT repair capacity, since even in the high alkyltransferase-expressing strain CHO-9-ada-C4 (generated by transfection with the bacterial ada gene) a significant residual level of mutations remained (see Table I for level of protection). Thus, after ENU exposure, most mutations are clearly caused by $O^6$-alkylguanine. A minor amount (10–25%) seems to be due to $O^4$-ethylthymine and very likely also to other lesions that are not repaired by MGMT and the Ada protein.

The alkyltransferase represents the major protective mechanism directed against alkylation-induced gene mutations. The alkyltransferase is probably also decisive in avoidance of spontaneous mutations both in bacteria [Rebeck and Samson, 1991] and in mammalian cells [Aquilina et al., 1992]. The conclusions drawn from work with the MGMT and ada transfectants support the concept that $O^6$-alkylguanine is the principal premutagenic lesion for MAA. This concept was initially based mainly on the findings that i) $O^6$-MeG mispairs with thymine during replication [Abbott and Saffhill, 1979], ii) most of the point mutations induced by MNNG, MNU, ENU and related drugs are GC → AT transitions (as detected, e.g., in the activated c-Ha-ras gene and the vermilion gene of D. melanogaster which is neutral for selection), and iii) there is a good correlation between the ability of MAA to react with the oxygen of bases in DNA, their efficiency to induce $O^6$-alkylguanine and their point mutation-inducing potency [Vogel et al., 1990, and further references herein].

## EFFECT OF ALKYLTRANSFERASE EXPRESSION ON SCE INDUCTION: AGENT SPECIFICITY OF THE CONTRIBUTION OF $O^6$-ALKYLGUANINE

At the chromosomal level SCEs are a manifestation of homologous recombination between the DNA molecules of a replicated chromosome, the formation of which is bounded on the passage of cells through the S phase. It is well established that all DNA damaging treatments induce SCE formation, albeit with different efficiencies. Cross-linking agents such as mitomycin C are quite efficient inducers, suggesting that replication-blocking lesions are causally involved in SCE formation. $O^6$-MeG and $O^6$-EtG are misinstructive lesions that do not (completely) block DNA replication. It was therefore interesting to note that SCE induction is dramatically reduced in pSV2MGMT transfected cells that express MGMT, as compared to the corresponding MGMT deficient neo$^+$ transfectant (or the non-transfected parent line CHO-9) (Fig. 1). There was no significant effect of MGMT expression on the spontaneous level of SCEs [see Kaina et al., 1991b]. SCEs induced by a treatment of 0.75 μM MNNG (1 h exposure) dropped from 46 exchanges in the control (strain CHO-9-neo-C5) to 8–14 exchanges per cell in high-level MGMT expressors. Taking into account the spontaneous SCE levels (see Table II legend) in transfectants that express more than 40000 MGMT molecules/cell

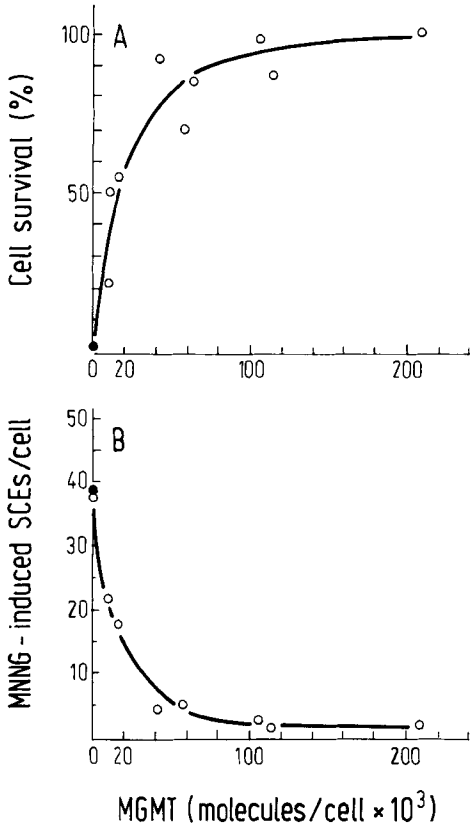


**Fig. 1.** MNNG-induced cell killing **(A)** and MNNG-induced SCE frequency **(B)** as a function of the level of alkyltransferase expressed in CHO-9 control ($Mex^-$) and various stable MGMT transfectant strains. In the survival experiments, cells were treated with 3 μM MNNG (60 min). The number of colonies appearing after 7 days is given in relation to the non-treated control. For a comparison, in (B) SCE frequency in relation to MGMT expression is shown (data from Kaina et al. [1991b] and unpublished). The SCEs were induced by treatment with 0.75 μM MNNG (60 min). The mutagen-induced SCE frequencies were calculated by subtracting the spontaneous SCE level from that obtained after MNNG treatment.

there was, on average, a residual induced level of 4 SCEs remaining per cell (see Fig. 1B). From these data we concluded that the vast majority of SCEs induced by MNNG is due to $O^6$-MeG, and that a minority of about 10% is caused by one or several other alkylation lesions [Kaina et al., 1991b].

A comparison of SCE frequencies (shown in Table II) induced by various MAA in CHO $Mex^-$ and MGMT or Ada expressing transfectants revealed remarkable differences in the degree of protection mediated by the alkyltransferase. These agent-specificities in protection against SCE formation indicate that the conclusion drawn above that $O^6$-alkylguanine is the major SCE-inducing lesion is true only for MNNG and MNU. Both agents induce nearly the same pattern of DNA alkylation [~9% of total alkylations in vivo occur at the $O^6$-position of guanine; see Beranek, 1990]. Assuming that SCEs induced by MAA are mainly due to $O^6$-alkylguanine, it is expected that the protection level correlates with the relative amount of $O^6$-alkylguanine they induce. This, however, is not the case. Thus ENU, which induces the highest relative amount of $O^6$-alkylguanine, yields only a slightly reduced SCE level in MGMT and ada transfected cells as compared to the $neo^+$ control (Table III). The level of protection is also significantly lower for EMS and for MMS than for MNNG and MNU (Table IV). These results suggest that in the case of MMS and the ethylating agents EMS and ENU, one or various lesions other than $O^6$-alkylguanine significantly contribute to SCE formation. The nature of these lesions is unknown. It should be noted that ENU induces an exceptionally high proportion of ethylphosphotriester in DNA (about 56%, as compared to 15% for MNU) which could be speculated to cause SCE formation. This lesion, however, and also $O^4$-ethylthymine are probably not involved in SCE induction since the expression of the Ada protein is as ineffective as MGMT in reducing the ENU-induced SCE frequency (see Table III).

## RELATION BETWEEN SCE AND KILLING PROTECTION MEDIATED BY THE ALKYLTRANSFERASE

$O^6$-Methylguanine induced in $Mex^-$ cells is clearly a toxic lesion, since MGMT expression in CHO transfectants (and HeLa transfectants, unpublished results) prevents MNNG and MNU-induced cell killing [Dunn et al., 1991; Kaina et al., 1991b; Wu et al., 1991]. It is remarkable that the survival of cells treated with MNNG increases as a function of MGMT activity, while at the same time the SCE frequency declines (Fig. 1). With >40,000 MGMT molecules/cell, protection against killing reaches a plateau at ~90% cell survival and SCE frequency is reduced by ~90%. Interestingly, complete prevention of both MNNG-induced killing and DNA recombination was not observed even in high MGMT expressing strains.

The correlation between SCE and killing protection suggests that SCE induction and reproductive cell death are interrelated phenomena. This conclusion gains support from the protection level mediated by MGMT against various alkylating agents. For the end point cell killing no significant protection was afforded against ENU, weak protection against MMS and EMS, and strong protection against MNNG and MNU. The correlation between killing and SCE protection levels for these MAA is shown in Figure 2. It indicates a similar relative contribution of $O^6$-alkylguanine to induction of SCEs and reproductive cell death for each reagent. It should be noted that there is no apparent correlation between the protection level and the electrophilicity of the compounds (see Table IV).

Comparing the efficiency of various MAA to induce SCEs and cell killing. Morris et al. [1982] also found a correlation between both end points. Since alkylation-induced SCEs and cell killing effects are obviously interrelated the question arises whether the recombinations giving rise to SCEs are toxic events, or whether $O^6$-MeG persisting

**TABLE II. Sensitivity of Strains CHO-9-Neo-C5, the MGMT Transfectant Tk40-AT8 and the Ada Transfectant Ada-C4 Towards the Induction of SCEs by Various Alkylating Agents**

| | Mutagen induced SCEs per cell[a] | | | | |
|---|---|---|---|---|---|
| Cell strain | MNNG (1 μM) | MNU (100 μM) | MMS (0.3 mM) | EMS (4 mM) | ENU (0.5 mM) |
| CHO-9-neo-C5 | 50.8 | 39.3 | 38.1 | 21.0 | 11.1 |
| Tk40-AT8 | 6.0 | 3.3 | 18.3 | 9.7 | 9.5 |
| CHO-9-ada-C4 | 8.9 | 4.8 | 25.1 | 10.2 | 10.9 |

[a]SCEs of the non-mutagen-treated cells (controls) were subtracted from those obtained after mutagen treatment. The spontaneous SCE levels were for strain neo-C5, 6.6 ± 2.2; Tk40-AT8, 6.2 ± 2.0; and ada-C4, 6.2 ± 2.2. For comparison, the nontransfected CHO-9 cells had a spontaneous SCE level of 5.9 ± 1.7.

**TABLE III. Induction of SCEs in MGMT Deficient CHO Cells (Strain CHO-9-neo-C5), the MGMT Transfectant Tk47-AT17-C3, and the Ada Transfectant Ada-C4***

| ENU concentration | CHO-9-neo-5 | Tk47-AT17-C3 | CHO-9-ada-C4 |
|---|---|---|---|
| control | 6.8 ± 2.3 | 9.2 ± 2.2 | 7.8 ± 2.8 |
| 0.8 mM | 22.9 ± 4.3 | 22.0 ± 5.4 | 26.6 ± 6.4 |
| 1.2 mM | 40.2 ± 10.6 | 29.9 ± 7.2 | 32.7 ± 6.3 |
| 1.6 mM | 55.8 ± 10.4 | 36.8 ± 8.1 | 41.2 ± 8.8 |

*ENU treatment was performed for 60 min. 25 metaphases were scored per treatment level. The transfectant Tk47-AT17-C3 expressed ~$2 \times 10^5$ MGMT molecules per cell. For MGMT level of the other strains see Table I.

in DNA exerts toxicity by another mechanism that does not involve DNA recombination. Further studies on interference of $O^6$-MeG with enzymes involved in DNA replication and recombination will hopefully clarify this question.

## IS $O^6$-METHYLGUANINE A CLASTOGENIC LESION?

Previously it has been shown that transfection of $Mex^-$ cells with the ada gene rendered them more resistant to aberration formation after alkylation [White et al., 1986; Kaina et al., 1991a]. This also holds true for $Mex^-$ Chinese hamster cells that were stably transfected with the human MGMT cDNA; these cells exhibited a lower level of MNNG and MNU-induced aberrations than the isogenic controls transfected with the neo gene only [Kaina et al., 1991b]. Protection was observed for all types of aberrations (Table V), indicating that $O^6$-MeG is involved in the generation of different types of chromosomal structural changes.

Based on the finding that after 1 h treatment with MNNG and a subsequent recovery time of 16 h, the aberration frequency was reduced by maximally 40% (from 70 to 40% aberrant metaphases), in MGMT expressing strains, the conclusion was drawn that the majority of MNNG-induced aberrations is due to lesions other than $O^6$-MeG [Kaina et al., 1991b]. Extending these studies with MGMT and ada transfectants we have found that the level of clastogenic protection exerted by the alkyltransferase increased with elevated recovery time (in preparation). On the basis of the mean aberration frequency induced in first and second post-treatment mitoses (recovery up to 36 h) the MNNG-induced aberration frequency in MGMT expressing cells is 88% lower than in the $Mex^-$ control. Thus the overall contribution of $O^6$-MeG to aberration formation in $Mex^-$ cells appears to be higher than previously concluded on the basis of data obtained with a recovery time of 16 h.

The clastogenic protection is not only dependent on the level of MGMT expression but also on the agent used. The MGMT transfectants proved to be most resistant to the clastogenic effect of MNNG and MNU, less resistant to MMS and EMS, and virtually no protection was observed against ENU-induced aberrations [Kaina et al., 1991b].

The strong contribution of $O^6$-MeG to aberration formation in case of MNNG and related agents, as deduced from the work with MGMT transfectants, apparently contradicts the conclusions drawn previously from a comparison of the s-value of MAA and their clastogenic potency in *Drosophila melanogaster*. Since agents with high s-value (such as MMS) were most clastogenic (as measured by X-ring chromosome formation in relation to sex-linked recessive lethal mutations), N-alkylations were suggested to be critical for induction of chromosomal breakage [Vogel et al., 1990]. MMS is in fact also highly clastogenic in mammalian cells, and the weak protection brought about by MGMT against MMS-induced clastogenicity indicates that N-methylpurines are indeed important for aberration production. We should stress the point that the *Drosophila* cells that were the target in the above-mentioned mutagenicity studies, are very likely able to repair $O^6$-MeG. It is therefore reasonable to suppose that most of the induced $O^6$-MeG was repaired in these cells before replication. Therefore the preponderant clastogenic lesions in case of MMS and related drugs might be the N-alkylpurines. In summary, the clear protective effect exerted by MGMT against MNNG and MNU-induced aberrations suggest $O^6$-MeG, if persisting in DNA, to be an important clastogenic lesion. The agent specificity of clastogenic protection and the correlation analyses furthermore suggest that at least two classes of alkylation lesions are

**TABLE IV.** *Level of Protection Caused by the Alkyltransferase Against SCEs Induced by Various Alkylating Agents, the Relative Amounts of O⁶-Alkylguanine They Induce in DNA, and Their Ability to Induce SCEs in the First and Second Post-Treatment Mitosis, Respectively*

| Agent | s-value[a] | $O^6$-AlkGua (%)[b] | SCE protection (%)[c] | SCEs first and second cell cycle[d] / SCEs second cell cycle |
|---|---|---|---|---|
| MMS | 0.83 | 0.3 | 52 | 46.6:23.2 |
| MNU | 0.42 | 8.2 | 92 | 38.4:3.4 |
| MNNG | 0.42 | 9.2 | 88 | 28.0:0.8 |
| EMS | 0.67 | 2.6 | 54 | 32.4:11.4 |
| ENU | 0.26 | 12.7 | 15 | 9.8:2.8 |

[a]Data from Vogel et al. [1990].
[b]Relative amount of $O^6$-alkylguanine induced in cellular DNA in in vivo, expressed as % of total alkylation; data from Beranek [1990].
[c]Reduction of mutagen-induced SCE frequency in MGMT expressing CHO cells (strain Tk40-AT8), as compared to the Mex⁻ control (strain CHO-9-neo-C5). For induced SCEs and mutagen concentrations see Table II.
[d]Mutagen-induced SCE frequencies of cells treated with the mutagen (for 60 min) two and one cell cycle before fixation, respectively. For mutagen concentrations and experimental details see Kaina and Aurich [1985].

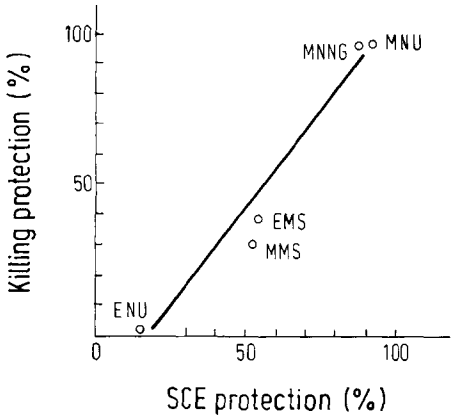


**Fig. 2.** Correlation between the effect of MGMT expression on cell killing and SCEs induced by various monofunctional alkylating agents. The killing protection mediated by MGMT was calculated from survival curves [see Kaina et al., 1991b] and given by the increase in cell survival of MGMT expressors (strain Tk40-AT8), relative to the Mex⁻ control (strain CHO-9-neo-C5), at a dose that reduced survival of the Mex⁻ control to the 10% level. SCE protection was calculated from data shown in Table II and expressed as the percentage of reduction of induced SCE frequency in the MGMT transfectant Tk40-AT8.

clastogenic. If the amount of potentially clastogeniclesion(s) other than $O^6$-MeG (possibly N-alkylpurines and the resulting apurinic sites) exceeds a particular threshold tolerated by the cells, these lesions seem to become most critical for aberration formation.

## CELL CYCLE DEPENDENCE OF CONVERSION OF $O^6$-METHYLGUANINE INTO SCEs AND ABERRATIONS: THE ROLE OF PRIMARY AND SECONDARY DNA LESIONS

Previously we have shown that the induction of most of the SCEs by MNNG and MNU in Mex⁻ Chinese hamster cells requires two rounds of replication after alkylation [Kaina and Aurich, 1985]. Thus treatment of cells with the same dose of MNNG one and two cell cycles before fixation induced 0.8 and 28 SCEs/cell, respectively. These experiments also revealed a remarkable agent specificity. Thus MMS, EMS and ENU induced significantly more SCEs in the first post-treatment replication cycle than MNU and MNNG (see Table IV). From these data we have concluded that both primary and secondary DNA lesions can give rise to SCEs. Most of the MNNG and MNU-induced SCEs are caused by secondary lesions that were introduced in DNA during the first replication cycle after alkylation. In this case the decisive critical primary lesion appears to be $O^6$-MeG since MGMT expression nearly completely prevented SCE induction. Those SCEs induced in the first post-alkylation replication cycle (a considerable part for MMS, EMS and ENU) are supposed to be due to primary lesions that are different from $O^6$ alkylguanine. They are also different from $O^4$-alkylthymine and the s-stereoisomere of the methylphosphotriester, since both the MGMT and the Ada repair activity does not or only weakly protects cells from SCE induction by ENU, MMS and EMS [Kaina et al., 1991b, and Table IV]. Furthermore, we have shown that the yield of SCEs induced in the first replication cycle after treatment with high doses of MNNG is not affected by MGMT expression (in preparation), suggesting that in this case other lesions apart from $O^6$-MeG are involved.

There are two lines of arguments indicating both primary and secondary DNA lesions to be also involved in aberration formation. Firstly, the protective effect exerted by MGMT and Ada on MNNG-induced clastogenicity is higher in the second than in the first post-treatment mitoses (in preparation). Secondly, about 5- to 10-fold higher doses of MNNG or MNU are required to induce aberrations in the first than in the second post-treatment mitoses [Thust et al., 1980;

**TABLE V. Frequency and Types of Aberrations Induced by MNNG (20 μM, 60 Min Treatment) in CHO-9, CHO-9-neo-C5, CHO-ada-C4 and Tk47-AT17-C3***

| Cell strain | No. mitoses scored | Aberrant metaphases (%) | Aberrations/ cell | g′ | g″ | b′ | b″ | Q | T | Dic | Exch |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CHO-9 | 100 | 73 | 1.21 | 1 | 3 | 24 | 1 | 19 | 34 | 2 | 37 |
| CHO-9-neo-C5 | 100 | 74 | 1.40 | 11 | 2 | 18 | 3 | 15 | 26 | 4 | 51 |
| CHO-ada-C4 | 100 | 4 | 0.20 | 7 | 0 | 0 | 0 | 0 | 1 | 0 | 12 |
| Tk47-AT17-C3 | 100 | 41 | 0.70 | 6 | 2 | 15 | 9 | 3 | 8 | 3 | 24 |

*g′, chromatid gaps; g″, isochromatid gaps; b′, chromatid breaks; b″, isochromatid breaks; Q, quadriradials; T, triradials; Dic, dicentrics; Exch, all other types of reunited and non-reunited exchanges.

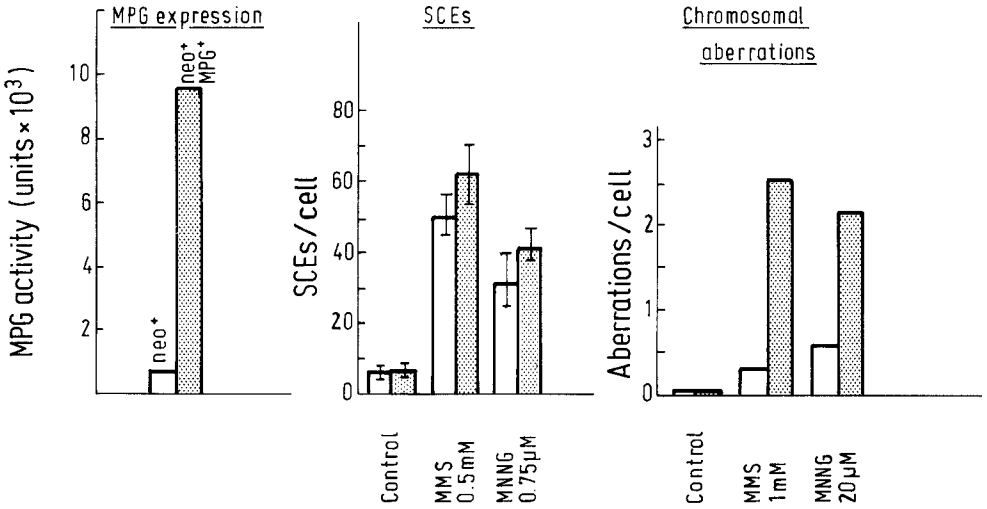


**Fig. 3.** N-methylpurine-DNA glycosylase (MPG) activity and SCE and aberration frequencies induced by MMS or MNNG in neo$^+$ (strain CHO-9-neo-C5) and neo$^+$MPG$^+$ (strain CHO-9-MPG-C9) transfectants. For a description of generation and further properties of the transfectant strains see Ibeanu et al. [1992].

Kaina, 1985]. This might indicate that most of the aberrations induced in the first post-alkylation replication cycle are due to N-alkylations which are tolerated at a low dose level, and that most of the aberrations induced in the second post-alkylation cell cycle are due to secondary lesions that were derived from $O^6$-alkylguanine persisting in DNA.

## DOES INCREASED REMOVAL OF N-METHYLPURINES FROM DNA PROTECT CELLS FROM SCE AND ABERRATION INDUCTION BY ALKYLATION?

If N-methylpurines (i.e., 7-MeG, 3-MeA, and 3-MeG) give rise to induction of SCEs and chromosomal aberrations, their increased removal from DNA is then supposed to render cells more resistant to MAA. To prove this hypothesis, transgenic Chinese hamster cell lines were generated that overexpress the N-methylpurine-DNA glycosylase (MPG) upon transfection with human MPG-cDNA [cloned by Chakravarti et al., 1991]. Although the transfectants expressed up to 16 times more MPG than the corresponding controls (CHO-9 or CHO-9-neo transfectant) and removed both 7-MeG and 3-MeA from DNA at a higher rate, they did not respond to MNNG and MMS treatment with a reduced level of SCEs and chromosomal aberrations. Instead the SCE frequency was significantly enhanced, and especially at high dose levels, cells showed a dramatic increase in chromosomal aberrations (Fig. 3). With high MMS doses there was also a marked reduction of cell survival, as compared to the non-MPG overexpressing control line [Ibeanu et al., 1992]. Apparently, increased removal of 7-MeG and 3-MeA from DNA in CHO cells is disadvantageous rather than helpful in avoiding genotoxic effects. The simplest explanation is that MPG is not rate limiting in excision repair of N-methylpurines in CHO-9 cells. MPG overexpression is supposed to give rise to a high yield of AP sites that are reported to be genotoxic [Loeb, 1985]. To provide an explanation for the fact that MPG overexpressing cells reacted more sensitively with increasing mutagen doses, it is proposed here that at high level of DNA alkylation, AP sites are generated in overlapping repair patches. If these are processed by AP endonuclease and exonuclease, DNA double-strand breaks are ultimately formed that give rise to chromosomal changes and toxic effects, and that may also initiate DNA recombination, leading to SCEs.

**TABLE VI. Probability that $O^6$-Methylguanine Induced in DNA of $Mex^-$ CHO Cells by MNNG Is Converted Into Sister Chromatid Exchanges or Chromosomal Aberrations (Transition Probability)**

| | | Induced events per cell in strains | | | | | | |
|---|---|---|---|---|---|---|---|---|
| End point | MNNG-treatment | Induced $O^6$-MeG per $10^7$ nucleotides[a] | Induced $O^6$-MeG per genome[b] | CHO-9-neo-C5 ($neo^+/MGMT^-$) | Tk47-AT17-C3 ($neo^+/MGMT^+$) | % reduction in $Mex^+$ cells | Corrected effect induced by $O^6$-MeG[c] | Transition probability |
| SCE | 0.75 μM, 60 min | 1.23 | 1,070 | 39.0 | 2.5 | 94 | 36.5 | 1:30 |
| Aberration | 15 μM, 60 min 16 h recovery | 23.7 | 20,550 | 0.21 | 0.07 | 67 | 0.14 | 1:147,000 |
| | 15 μM, 60 min 36 h recovery | 23.7 | 20,550 | 0.94 | 0.01 | 89 | 0.93 | 1: 22,000 |

[a]Data obtained from linear extrapolation of dose-effect-curve of induced $O^6$-MeG in CHO-9 cells [Kaina et al., 1990];
[b]Based on a DNA content of CHO cells in G1 of 4.78 pg, corresponding to $8.67 \times 10^9$ nucleotides [Sontag et al., 1990].
[c]SCEs and aberrations that are supposed to be due to $O^6$-MeG in $Mex^-$ CHO cells; data were obtained by correction of SCE and aberration frequencies induced in CHO-9-neo-C5 cells on the basis of the percentage of reduction observed in cells expressing MGMT at high level (strain Tk47-AT17-C3).

## PROBABILITY OF CONVERSION OF $O^6$-MeG INTO SCEs AND ABERRATIONS

If all the protective effects observed in MGMT transfectants were due to repair of $O^6$-MeG (which is very likely the case, as even if $O^4$-MeT is repaired by MGMT, its contribution can be neglected because of the low amounts induced) what then is the probability of conversion of this lesion during replication into various end points? A dose of 0.75 μM of MNNG induces 39 SCEs/$Mex^-$ cell and about $10^3$ $O^6$-MeG lesions per Chinese hamster genome. If >90% of the induced SCEs are due to $O^6$-MeG, as deduced from the MGMT mediated protection level, then ~30 $O^6$-MeG molecules induced and persisting in the genome are expected to give rise to one SCE per CHO cell (see Table VI).

The same dose reduced cell survival by ~40% in CHO $Mex^-$ cells and had no effect on survival of MGMT expressing variants. Apparently a small number of $O^6$-MeG molecules induced per genome is sufficient for SCE induction and cell inactivation. This is in line with a previous report in which, for some $Mex^-$ cell lines, 280–500 $O^6$-MeG molecules were calculated to be induced in DNA by MNNG at $D_{37}$ dose level [Karran and Bignami, 1992]. Furthermore, the calculations agree reasonably well with a previous estimation for $Mer^-$ human tumor cells for which 2–20 $O^6$-MeG molecules were reported to correspond to 1 SCE [Scudiero et al., 1984].

Similar calculations were performed by us for chromosomal aberrations (see Table VI for MNNG and a recovery time of 16 and 36 h). The probability of $O^6$-MeG being converted into aberrations is much lower than into SCEs. Thus about 147,000 $O^6$-MeG molecules are required per genome of a $Mex^-$ cell for the generation of one aberration in the first post-treatment cell cycle. For the consecutive cell cycle the amount of $O^6$-MeG that gives rise to an aberration event is significantly lower; it has been estimated to be in the range of 22,000 molecules/cell.

## CONCLUSIONS

$O^6$-methyl- and $O^6$-ethylguanine are the major point-mutagenic lesions for MAA. The contribution of these lesions to the formation of SCEs and chromosomal aberrations appears to be dependent or the absolute amounts present in DNA during replication in relation to other alkylation damages. In cells that do not repair $O^6$-MeG ($Mex^-$), this lesion is most important for SCE and aberration formation in case of treatment with MNNG and MNU. For other alkylating agents such as MMS, EMS and ENU, and for cells that are phenotypically $Mex^+$, lesions other than $O^6$-alkylguanine appear to contribute significantly to SCE and aberration formation, as well as to toxic effects. Since N-methylpurine-DNA-glycosylase overexpressing cells are sensitized to methylating agents rather than gaining resistance, it is concluded that apurinic sites resulting from repair of N-methylpurines contribute to the toxic, SCE-inducing and clastogenic effects of MMS and related drugs as well.

$O^6$-MeG is not a replication blocking lesion. Nevertheless a very low quantity (~30 molecules per CHO $Mex^-$ cell) already appears to be sufficient for the induction of SCEs and cell killing. Considerably higher quantities persisting in DNA are necessary for induction of chromosomal aberrations. $O^6$-MeG is probably converted into a secondary lesion during replication since efficient induction of SCEs and aberrations by MNNG and MNU needs two rounds of replication. The nature of the secondary lesions that are postulated here to be involved in generation of most of the genotoxic effects induced by MNNG and MNU remains to be elucidated.

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